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R C van Dijk

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RMF Dictagene S.A.
Chemin de la Vulliette 4
1000 Lausanne
SUISSE

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Angiogenesis inhibiting molecules, their selection, production and their use in the treatment and diagnosis of cancer

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ANGIOGENESIS INHIBITING MOLECULES, THEIR SELECTION, PRODUCTION AND THEIR USE IN THE TREATMENT AND DIAGNOSIS OF CANCER.

The present invention relates to a method for providing molecules that are capable of inhibiting angiogenesis, to molecules thus provided, to therapeutical or diagnostic compositions comprising one or more of the molecules and to the use of such molecules in the treatment or diagnosis of cancer, in particular solid tumors.

Angiogenesis, the formation of new blood vessels from the pre-existing vasculature is fundamental to wound healing, reproduction and embryonic development. Angiogenesis is also essential for the development of tumors. New blood vessels in tumors provide nutrients allowing the cells to undergo uncontrolled mitosis.

During angiogenesis, endothelial cells proliferate, migrate into new tissue and form inter-endothelial junctions leading to tube formation. This process starts and is driven by angiogenic factors. The signalling of VEGFs and angiopoietins leads to loosening of the pericyte-endothelial contact permitting proliferation and interaction of new endothelial cells with the extracellular matrix mediated by integrins. The $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins have been described to participate in blood vessel development and angiogenesis via a signalling crosstalk with angiogenic factors.

In addition to interactions between endothelial cells and the extra-cellular matrix, the regulation of inter-endothelial contacts is important for tube formation. For example, the adhesion molecule VE-cadherin plays a role in vascular remodelling and maintains integrity of blood vessels.

In the research that led to the present invention the junctional adhesion molecules JAM-B and JAM-C were discovered. These molecules are found in vascular cell-cell contacts and are involved in leukocyte transendothelial migration. Now it was found that the interaction between JAM-B and JAM-C also plays an important role in angiogenesis.

Based on this finding it is the object of the present invention to provide new molecules that are capable of inhibiting angiogenesis.

This is achieved by the present invention by a method, comprising the steps of:

a) providing a range of molecules;

b) testing whether these molecules can prevent interaction between JAM-B and JAM-C;

c) testing the positive molecules for their ability to block angiogenesis *in vivo*; and

d) selecting molecules that are positive in the *in vivo* test as angiogenesis inhibiting molecules.

Testing the molecules for their ability to block angiogenesis *in vivo* may be performed by means of the retina test as described in Example 4.

According to the invention it was found that not all molecules that can prevent the interaction between JAM-B and JAM-C are also capable of inhibiting angiogenesis. The additional test of step d) above is therefore necessary to find the desired molecules.

In another embodiment the invention may further comprise the step of testing the positive molecules for their ability to inhibit tumor growth *in vivo*. The test for inhibiting tumor growth *in vivo* is for example a test as described in Example 5.

The method of the invention may further comprise the step of isolating or producing the angiogenesis inhibiting

molecules thus resulting in the actual obtainment of the desired molecules.

The range of molecules that can be tested according to the invention can be diverse. In a first embodiment, the range of molecules is a population of antibodies directed against JAM-B or JAM-C. The preparation of antibodies is a straightforward technique not requiring inventive skill and for example described in Köhler & Milstein, Nature 256:495-497 (1975). The skilled person will therefore be able to provide such range without undue burden.

Once the desired antibody is selected it can be produced by means of well known techniques. Provision of the actual angiogenesis molecule is therefore straightforward.

In a second embodiment the range of molecules is a population of small molecules binding to JAM-B or JAM-C. Small molecules (molecular weight under 1,000 daltons) include but are not limited to sugars, amino acids, lipids, vitamins, hormones and chemicals. In the field of biotechnology "small molecule" is a well-understood term. In a preferred embodiment the small molecules are unnatural chemical compounds. Because the small molecules that are tested are already physically existent, the further step of producing the selected small molecule does not require inventive skill.

Provision of a population of small molecules is for example described in Thompson & Ellman, "Synthesis and Applications of Small Molecule Libraries", Chem. Rev. 96, 555-600 (1996); E. Jacoby et al., "Design of Small Molecule Libraries for NMR Screening and Other Applications in Drug Discovery", Current Topics in Medicinal Chemistry 3(1), 11-23 (2003); and Schreiber, S.L. "The small-molecule approach to biology: Chemical genetics and diversity-oriented organic

synthesis make possible the systematic exploration of biology", Chem. & Eng. News. 81, 51-61 (2003). Further informations on such libraries can be found for example at the following sites:

http://www.neogenesis.com/chemgen/technology.html
http://www.small-molecule-drug-discovery.com/high_screening.html
http://thomsoncurrentdrugs.com/ebulletin/stemcell/
http://www.scripps.edu/news/press/060203.html
http://www.pharmacopeia.com/corp/cnews/pr/pr19970129.html
http://www.bu.edu/cmld/overview.htm
http://www.fbodaily.com/archive/2003/10-October/05-Oct-2003/FBO-00447362.htm

Testing whether molecules can prevent interaction between JAM-B and JAM-C is for example performed by incubating cells expressing either JAM-B or JAM-C on their surface with labelled soluble JAM-C or JAM-B, respectively, in the presence of said molecules and recording a decrease in labelling of the cells as compared to control incubation without said molecules. Molecules that induce a decrease in the amount of label visualized in comparison to control cells expressing JAM-C or JAM-B and having labelled JAM-B or JAM-C but no molecule to be tested bound to their surface are selected as positive molecules.

Suitable labels are fluorescent, radioactive or Biotin based labels that are well known in the art. Suitable techniques for visualizing the label and disappearance or reduction thereof are flow cytometry, biochemistry, or enzyme linked immunosorbent assay (ELISA).

Molecules that are found to inhibit the interaction between JAM-B and JAM-C are then further tested for their ability to inhibit angiogenesis *in vivo*. For this various options are available. However, the retina test as described

in Example 4 is very well suited because the remodelling of the vasculature depends essentially on endothelial cells and not on microenvironmental factors. Alternative tests are the chorio allantois membrane assay, ischemic reperfusion, or angiogenesis induced by graft of matrigel loaded with angiogenic factors. These tests are well known in the art.

In addition to or instead of testing the ability to inhibit angiogenesis *in vivo*, the capability of the molecule to inhibit tumor growth *in vivo* may be tested. A suitable example of such test is as described in Example 5.

The invention according to a further aspect thereof relates to angiogenesis inhibiting molecules selected according to the method of claim 1 and subsequently produced.

Such molecules may be antibodies or small molecules.

The invention further relates to antibody fragments and antibody derivatives that retain the antigen binding capacity of the whole antibody. Functional antigen-binding antibody fragments can be engineered by proteolysis of antibodies (papain digestion, pepsin digestions or other enzymatic approaches), yielding Fab, Fv or single domains. Alternatively, fragments can be produced recombinantly. Fab fragments ("Fragment antigen binding") are the antigen-binding domains of an antibody molecule, containing V_H + C_H1 and C_L + V_L. Between C_L and C_H1 an interchain disulfide bond is present. The molecular weight of the heterodimer is usually around 50 kDa. Fab fragments can be prepared by papain digestions of whole antibodies. The minimal fragment (~30 kDa) that still contains the whole antigen-binding site of a whole IgG antibody is composed of both the variable heavy chain (V_H) and variable light chain (V_L) domains. This heterodimer, called Fv fragment (for "Fragment variable") is still capable of binding the antigen. Another fragment is the single domain antigen binding fragment (dAbs) or V_Hs. Single-

chain Fv fragments can be made recombinantly. In the scFv fragment the V_H and V_L domains are joined with a hydrophilic and flexible peptide linker. scFvs can be complexed into dimers (diabodies), trimers (triabodies) or larger aggregates the monomeric units of which can have the same or different specificities. A further type of antibody fragment are the V_{HH}s comprising the smallest available intact antigen-binding fragment. V_{HH}s can be produced from proteolysed heavy chain antibodies of an immunised camelid or via recombinant techniques. Antibodies can furthermore be humanized, such as complementarity-determining region (CDR)-grafted, and chimeric. These and other known or future antibody fragments or antibody derivatives having the ability to prevent the interaction between JAM-B and JAM-C and to inhibit angiogenesis *in vivo* are part of this invention. The production of antibody fragments and antibody fusion proteins is reviewed in Joosten, V. et al., *Microb Cell Fact.* 2(1): 1 (2003). Techniques for preparing antibody fragments and derivatives are widely known and can be performed by the person skilled in the field without undue burden.

Also provided by the invention are hybridoma cell lines that produce monoclonal antibodies reactive with either JAM-B or JAM-C polypeptides.

In a particular embodiment the invention relates to the monoclonal antibody H33, produced by hybridoma 13H33, deposited on 22 October 2003 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the deposit accession number **DSM ACC2622**. It was shown that antibody H33, which is directed against JAM-C, can block angiogenesis *in vitro* and *in vivo* and prevent tumor growth *in vivo*. It can also block the interaction of JAM-C with JAM-B. Hybridoma 13H33 is also part of this invention.

The invention relates to the antibody H33 for use as a medicament, in particular for treatment of cancer, more in particular for treating solid tumors. In addition the invention relates to fragments and derivatives of H33, in particular Fab fragments, Fv fragments, single domain antigen binding fragments, recombinant antibodies having the specificity of H33, scFv and aggregates thereof, V_{HH}s, humanized derivatives of H33 and chimeric antibodies comprising at least the specificity of H33.

According to another embodiment the invention relates to the use of soluble JAM-B or JAM-C as a medicament, in particular for treatment of cancer, more in particular for treating solid tumors.

The invention also relates to the use of the angiogenesis inhibiting molecules, such as H33, fragments and derivatives thereof, or soluble JAM-C or JAM-B for the preparation of a therapeutical or diagnostic composition for the treatment or diagnosis of cancer, in particular solid tumors.

The invention also relates to therapeutical or diagnostic compositions for the treatment or diagnosis of cancer, in particular solid tumors, comprising a therapeutically or diagnostically effective amount of one or more angiogenesis inhibiting molecules of the invention and a suitable excipient, carrier, diluent or other additive. The skilled person in the field of cancer therapy will be able to establish the therapeutically or diagnostically effective amount.

The invention thus also relates to the use of the compounds of the invention in diagnosis. Labeled antibodies can for example be used for locating tumors in the body. Labeling of antibodies with radioactive, paramagnetic or other is a technique well-known in the art.

The present invention will be further described in the Examples that follow. These Examples are for illustration purposes only and not intended to limit the invention in any way. In the Examples reference is made to the following figures.

Figure 1 shows that JAM-C is expressed by blood vessels in human liver tumor. JAM-C expression was analysed with a panel of angiogenic tumors. The transcripts encoding human JAM-C are not present in normal liver. Immunostaining of frozen sections with anti-JAM-C antibody shows expression by a subpopulation of blood vessels (arrowheads). Staining with a polyclonal antibody against PECAM-1 to visualize vascular structures is shown on the right panel and the angiogenic characteristic of the tumor was controlled by $\alpha v\beta 3$ staining (insert).

Figure 2 shows that JAM-C is recruited at inter-endothelial junctions of HUVECs upon VEGF stimulation. (A) HUVECs were stimulated with recombinant VEGF-165, fixed with formaldehyde and JAM-C localization was visualized with anti-JAM-C monoclonal antibody. As control, JAM-A staining was performed. The JAM-C molecule was enriched at cell-cell contacts upon VEGF-165 stimulation whereas no effect was seen with JAM-A. (B) FACS analysis revealed that the enrichement of JAM-C at cell-cell contacts is due to relocalization of the molecule since the expression level remained unchanged after VEGF treatment (thin line, negative control; dashed line, non treated cells; thick line, VEGF treated cells).

Figure 3 shows that anti-JAM-C monoclonal antibody abolished angiogenesis *in vitro*. Aortic rings from mice were grown between two Matrigel layers in the presence or absence of anti-JAM-C antibodies (50 µg/ml) and neovascularization was visualized after 12 days. Pictures are light micrographs of representative non treated (A, n=11) or treated aortic

ring microvessels with anti-JAM-C monoclonal antibodies H33 (B, n=11) and D33 (C, n=6) or isotype-matched control antibody Mel14 (D, n=6). Only H33 blocked angiogenic sprouting.

Figure 4 demonstrates that anti-JAM-C antibody H33 reduced tumor growth and tumor vascularization. Mice were injected sub-cutaneously with LLC1 tumor cells and treated every second day with anti-JAM-C antibody or isotype-matched control antibody (150 µg). (A) macroscopic appearance of 12-day-old LLC1 tumors grown in control mice (PBS and isotype-matched control antibody) or in mice treated with H33 anti-JAM-C antibody. Mice treated with H33 anti-JAM-C antibody show reduced tumor growth as indicated by measurement of tumor volume (B) and tumor weight (C). Microvessels were detected by PECAM-1 immunostaining (D) and quantified by computer analysis (E).* $p < 0,01$. Each bar represents the mean value of ten animals tested ± sem.

Figure 5 shows that H33 anti-JAM-C antibody is not toxic *in vivo*. To ensure that the H33 anti-JAM-C antibody effect on tumor growth was not due to a general toxic effect *in vivo*, mice were treated as described in **Fig. 4** and the organs dissected and analysed. (A) Kidney from these mice stained with periodic acid-Schiff (PAS) did not show any signs of glomerulonephritis development. As control, sections of kidney from autoimmune diseased NZBxBXSB mice were compared (Merino R. et al., *J Clin Invest.* 94(2):521-5 (1994). (B) *In vivo* blood vessel permeability was assessed using the Evans blue permeability assay. The H33 antibody had no effect on vascular permeability in the representative organs heart, lung, kidney and brain. Each bar represents the mean value of seven animals tested ± sem.

Figure 6 shows quantitation of glomeruli during revascularization of retinas. The numbers of glomeruli were

counted to compare retinal neovascularization in H33-treated and control antibody-treated mice. Reduction in the number of glomeruli was observed in H33 treated (13H33) compared to control mice (ctrl) or mice treated with isotype matched control antibody (9B5). This indicates a decreased neovascularization of retinas in H33 treated animals.

EXAMPLES

EXAMPLE 1

Preparation of a population of antibodies against JAM-B or JAM-C

A population of antibodies to be tested in the method of the invention is prepared according to Köhler & Milstein, Nature 256:495-497 (1975). The source of antigen to obtain such population of antibodies consist in recombinant soluble JAM-B or JAM-C prepared as described in Example 3.

EXAMPLE 2

Preparation of soluble JAM-B and JAM-C

It was found by the present inventors that JAM-C interacts heterophilically with JAM-B through its V domain and that the soluble JAM-C V domain is sufficient for binding to JAM-B.

The soluble JAM-B and the soluble JAM-C V domain were obtained by PCR using the same cloning strategy. Primers were obtained from Microsynth (Microsynth GmbH, Balgach, Switzerland), and restriction sites added for cloning strategy are underlined. The cDNA encoding the extracellular V domain of JAM-C was amplified using plasmid encoding the full length sequence of murine JAM-C, Pfu polymerase, T7 and (5'-gctctagacagtgttgccgtcttgcctacag-3') as forward and reverse primers. The PCR product was digested with HindIII and XbaI before cloning in pcDNA3 containing FLAG-tag

sequence.

The soluble JAM-B is prepared as follows: The cDNA encoding soluble JAM-B was obtained by PCR using (5'-tcagctaggcagccagct-3') and (5'-gctctagaatctacttgcattcgcttcc-3') as forward and reverse primers. The PCR product digested with XbaI was then cloned in frame with the FLAG Tag sequence in pcDNA3 using EcoRI/blunt and XbaI sites.

EXAMPLE 3

Test for the ability to prevent interaction between JAM-B and JAM-C

Cells expressing either JAM-B or JAM-C on their surface are obtained as described by Aurrand-Lions et al., *J Biol Chem* 276:2733-41 (2001a); Aurrand-Lions et al., *Blood* 98:3699-707 (2001b); Johnson-Leger et al., *Blood* 100:2479-2486 (2002).

Soluble JAM-B and JAM-C obtained as described in Example 2 are labelled with sulfosuccinimidyl esters of Alexa 488 (Molecular Probes Inc.) or sulfo-NHS-Biotin (Pierce) according to the manufacturers procedures.

The cells expressing JAM-B or JAM-C are contacted with the labelled soluble JAM-C or JAM-B, respectively in in the presence of the molecules to be tested. The fluorescence is monitored with flow cytometry and decrease in fluorescence intensity as compared to the non treated control, indicates a decreased binding of soluble JAM-C or soluble JAM-B.

EXAMPLE 4

Test for the ability to block angiogenesis *in vivo*

Postnatal day 7 (P7) mice are placed in 75% oxygen for five days causing central avascularization of retinas (Reynolds et al., *Nature Medicine* 8: 27-34 (2002)). This

incubation is followed by housing the mice for five further days (until P17) under normoxic conditions. Mice are injected intraperitoneally with 50 µg of monoclonal antibodies at P12, P14 and P16. Neovascularization is detected by perfusion of the entire vasculature with a non-diffusible fluorescein-dextran solution. In flat-mounted retinas, areas of neovascularization and vascular glomeruli are detected. Vascular glomeruli are highly proliferative clusters of tortuous vessels that are produced in response to angiogenic stimuli and protrude through the inner limiting membrane. The numbers of glomeruli is counted to compare retinal neovascularization in mice treated with a molecule to be tested and in control mice.

One of the molecules tested is monoclonal antibody H33 which caused a reduction in neovascularization.

EXAMPLE 5

Monoclonal antibody H33 directed against JAM-C is an inhibitor of angiogenesis and tumor growth

1. MATERIALS AND METHODS

Antibodies.

Rat monoclonal antibodies (CRAM) against human and mouse JAM-C (H33, H36 and D33) and rat monoclonal antibodies against mouse PECAM-1/CD31 (GC51) and Lselectin/CD62L (Mel14) were previously described (Aurrand-Lions et al., 2001a, supra; Gallatin et al., *Nature* 330:30-34 (1983); Piali et al., *Eur J Immunol*. 23:2464-71 (1993); Springer et al., *Eur. J. Immunol*. 9:301 (1979). Anti-human CD44 (9B5) used as irrelevant antibody control rat IgG2a was kindly provided by Dr B. Engelhardt (Laschinger and Engelhardt, 2000). Any other unrelated antibody can be used as a negative control. Polyclonal antibody against human JAM-B was prepared

according to Palmeri et al., *J Biol Chem.* 275:19139-45 (2000). Monoclonal mouse anti-human integrin αvβ3 (LM609) were from Chemicon (Temecula, CA).

Endothelial cells

Human Umbilical Vein Endothelial Cells (HUVEC) were isolated by collagenase treatment of umbilical veins (Wall RT et al., *J Cell Physiol.* 96:203-213 (1978). HUVECs were maintained in M199 supplemented with 20% Fetal Calf Serum (PAA Laboratories), 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), nonessential amino acids, sodium pyruvate, endothelial cell growth supplement (ECGS, 15 µg/mL; Upstate Biotechnology, Lake Placid, NY), and heparin (4 µg/ml; Sigma, Buchs, Switzerland). Cells were used between passages 3 and 5.

VEGF stimulation

1.10^5 HUVECs were plated on Growth Factor Reduced Matrigel (Becton Dickinson, Bedford, MA, USA). After 48 hours, cells were incubated with 100 ng/ml recombinant human VEGF-165 (PeproTech House, London UK) for 15 minutes (immunocytochemistry) or 15 minutes to 24 hours (Flow cytometry).

Flow cytometry

HUVECs were incubated with H36 anti-JAM-C monoclonal antibody on ice. After washing with PBS, BSA 0.2% binding of H36 antibody was detected using a phycoerythrin-coupled anti-rat antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA). As control, the primary antibody was omitted. Analysis was performed using FACSCalibur and Cellquest Software (Becton Dickinson, Mountain View, CA, USA).

Immunostaining

For immunohistochemistry with monoclonal antibody anti-JAM-C (H36) and polyclonal antibody against JAM-B/VE-JAM, frozen sections were fixed with acetone/methanol 1:1 for 5 minutes at -20°C, dried and rehydrated in PBS, Gelatin 0.2%, Tween 20 0.05%. For immunocytochemistry, cells were fixed with paraformaldehyde 4% in PBS for 15 min prior permeabilization with TritonX100 0.01% in PBS for 10 min. Cells were washed with PBS, BSA 0.2%, incubated with primary antibodies for one hour and washed, before further incubation with secondary antibodies coupled to Texas Red, FITC or peroxidase (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA). Pictures were acquired using confocal microscope Zeiss LSM510. Glomerulonephritis was detected by kidney staining with periodic acid-Schiff (PAS).

Ex vivo aortic ring assay

Mouse aortic ring assays were performed as described (Nicosia, R.F. & Ottinetti, *In Vitro Cell Dev Biol.* 26(2):119-28 (1990). Briefly, 1-mm thoracic aortic rings were placed between two layers of 50 µl growth factor-reduced Matrigel (Becton Dickinson, Bedford, MA, USA) optionally containing an antibody to be tested, and overlaid with 100 µl of DMEM supplemented with 20 U/ml heparin (Sigma-Aldrich corporation, Saint-Louis, MO, USA) and ECGS (Upstate biotechnology, Lake Placid, NY, USA). Microvessel outgrowth was visualized by phase microscopy using a Zeiss Axioskop microscope.

Tumor graft

Female 8- to 10- week-old C56BL6/J mice (Charles River laboratories, L'Arbresle, France) were inoculated subcutaneously with $1x10^6$ murine Lewis lung carcinoma

cells(LLC1). Mice were then injected intraperitoneally every second day with 150 µg of monoclonal antibody H33, isotype matched control antibody Mel14 or PBS. When the control tumors (PBS injected mice) had reached 1-1.5 cm^3 animals were sacrificed and tumors were excised and analysed.

Vessels quantification

Tumor cryosections were stained with monoclonal anti-PECAM-1 antibody as described in Immunostaining Chapter. Pictures of the entire cryosections (4 cryosections per tumor) were taken using a Zeiss Axioskop microscope. PECAM-1 staining and the total area of the tumor were quantified using Zeiss KS400 software.

Evans blue permeability assay

150 µg of anti-JAM-C or isotype-matched control antibodies were injected into the retro-orbital vein of anaesthetised mice. After 15 minutes, 100 µl of a 30mg/kg Evans blue dye (Sigma-Aldrich corporation, Saint-Louis, MO, USA) solution in saline was injected in the same way as antibodies, and circulated for one hour. Mice were then perfused with citrate-buffered 1% paraformaldehyde, pH 4.2, 37°C to clear the dye out of the vessel lumina. Immediately after perfusion, the organs (kidney, lung, heart and brain) were dissected. After drying (Speed-Vac) of the tissue, the dried weight was measured. Evans blue was extracted by subsequent incubation of the tissue in 500 µl of formamide for 18 hours at 70°C. The extract was centrifuged and the absorbance of the supernatant was measured at 620 nm with a spectrophotometer. The dye concentration in the extracts was calculated from a standard curve of Evans blue in formamide and normalized to the dry tissue weight.

Statistical analysis

Vessel density counts, tumor volume and tumor weight were analysed using the Mann-Whitney's *t* test. Analyses were computed using the statistical software StatView (Abacus Concepts Inc, Berkeley, CA, USA).

RESULTS

JAM-C is expressed by tumor vessels and is receptive to angiogenic stimuli

In angiogenic tumor of human liver, anti-JAM-C antibody H36 stains blood vessels (**Fig. 1**). In contrast, the transcript encoding JAM-C is not present in normal liver. Treatment of HUVECs with VEGF leads to immediate and massive accumulation of JAM-C in endothelial cell-cell contacts within 15 minutes (**Fig. 2A**). This short appearance is the result of JAM-C relocalization and the expression level is not modified by this treatment (**Fig. 2B**). The same results were observed when HUVECs were stimulated with TNF-α or thrombin.

In vitro vessel outgrowth is inhibited by anti-JAM-C monoclonal antibody

In vitro neovascularization was carried out using *ex vivo* aortic rings assays. Freshly dissected mouse aortae were cut into small rings and those embedded in Matrigel in the presence or absence of anti-JAM-C antibodies. Outgrowth of endothelial vessels from the aortic rings were assessed over a period of 12 days. Whereas the presence of control anti-JAM-C or isotype matched antibodies do not affect aortic sprouting, the H33 anti-JAM-C antibody totally blocks neovascularization (**Fig. 3**).

Anti JAM-C monoclonal antibody reduces tumor growth and angiogenesis in vivo

Given that *in vitro* angiogenesis can be blocked with H33 anti-JAM-C antibody, we investigated wether this antibody had an effect on tumor angiogenesis and tumor growth. Mice were sub-cutaneously injected with Lewis lung carcinoma cells. Anti-JAM-C and control antibodies were then injected intra-peritoneally every second day. Animals were sacrificed when the control tumors reached 1-1.5 cm^3 and tumors excised. Tumor size (Fig. 4A), volume (Fig. 4B) and weight (Fig. 4C) were significantly decreased when mice were treated with H33 anti-JAM-C antibody compared with the control isotype matched antibody or PBS.

Representative examples of excised tumors are shown in figure 4A. Lewis lung carcinoma cells do not express JAM-C (data not shown). The H33 antibody effect on tumor growth is due to inhibition of angiogenesis. In order to visualize the tumor vasculature, cryosections were immunostained with antibody against the endothelial marker PECAM-1 (**Fig. 4D**). Blood vessel density was quantified by counting the % of PECAM-1 staining across the area of the tumor (**Fig. 4E**). The H33 antibody reduced the number of blood vessels in tumors when compared to the controls.

H33 anti-JAM-C monoclonal antibody is not toxic in vivo

It is known that antibodies can be toxic when injected *in vivo*. In order to control that the observed effect of H33 anti-JAM-C antibody is not due to a general toxic effect in mice it was investigated whether antibody injected animals develop pathologies. Since JAM-C is expressed by endothelium in the kidney it was first analysed whether the antibody would create glomerulonephritis. To this end kidney sections of treated animals were stained with

periodic acid-Schiff. No abnormal accumulation of protein in glomeruli was detected (**Fig. 5A**).

Since JAM-C is involved in controlling vascular permeability it was also tested whether the antibody would induce leakiness of blood vessels. Fortunately this was not the case in heart, lung, kidney or brain, the representative organs analysed (**Fig. 5B**).

Reduction in the number of glomeruli was observed in the retinas of H33 treated compared to control mice (ctrl) or mice treated with isotype matched control antibody. This indicates a decreased neovascularization of retinas in H33 treated animals (**Fig. 6**).

CLAIMS

1. Method for providing molecules that are capable of inhibiting angiogenesis, comprising the steps of:

a) providing a range of molecules;

b) testing whether these molecules can prevent interaction between JAM-B and JAM-C;

c) testing the positive molecules for their ability to block angiogenesis *in vivo*; and

d) selecting molecules that are positive in the angiogenesis test as angiogenesis inhibiting molecules.

2. Method as claimed in claim 1, wherein the *in vivo* angiogenesis test is the retina test as described in Example 4.

3. Method as claimed in claim 1 or 2, further comprising the step of testing the positive molecules for their ability to inhibit tumor growth *in vivo*.

4. Method as claimed in claim 3, wherein the test for inhibiting tumor growth *in vivo* is as described in Example 5.

5. Method as claimed in any one of the claims 1 to 3, further comprising the step of isolating or producing the angiogenesis inhibiting molecules.

6. Method as claimed in any one of the claims 1 to 5, wherein the range of molecules is a population of antibodies directed against JAM-B or JAM-C.

7. Method as claimed in any one of the claims 1 to 5, wherein the range of molecules is a population of small molecules binding to JAM-B or JAM-C.

8. Method as claimed in claim 7, wherein the small molecules are unnatural chemical compounds.

9.Method as claimed in any one of the claims 1-8, wherein testing whether molecules can prevent interaction between JAM-B and JAM-C is performed by incubating cells

expressing either JAM-B or JAM-C on their surface with labelled soluble JAM-C or JAM-B, respectively, in the presence of said molecules and recording a decrease in labelling of the cells as compared to control incubation without said molecules

10. Angiogenesis inhibiting molecules obtainable by the method as claimed in claims 1-9.

11. Angiogenesis inhibiting molecules as claimed in claim 10, which molecules are antibodies, antibody fragments or antibody derivatives.

12. Angiogenesis inhibiting molecules as claimed in claim 11, wherein the antibody fragments are selected from Fab fragments, Fv fragments, single domain antigen binding fragments, scFv and aggregates thereof, V_{HH}s.

13. Angiogenesis inhibiting molecules as claimed in claim 11, wherein the antibody derivatives are recombinant antibodies having the same specificity as the selected antibody, humanized derivatives of the selected antibody and chimeric antibodies based on the selected antibody.

14. Angiogenesis inhibiting molecules as claimed in claim 10, which molecules are small molecules.

15. Angiogenesis inhibiting molecules selected from soluble JAM-B and soluble JAM-C.

16. Angiogenesis inhibiting molecule as claimed in any one of the claims 10-13, wherein the antibody is H33, produced by hybridoma 13H33 as deposited on 22 October 2003 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the deposit accession number **DSM ACC2622**.

17. Angiogenesis inhibiting molecule as claimed in any one of the claims 10-13 for use as a medicament.

18. Angiogenesis inhibiting molecule as claimed in any one of the claims 10-13 for use in the treatment or

diagnosis of cancer.

19. Angiogenesis inhibiting molecule as claimed in claim 18 for use in the treatment or diagnosis of solid tumors.

20. Use of angiogenesis inhibiting molecule as claimed in any one of the claims 10-13 for the preparation of a therapeutical or diagnostic composition for the treatment of cancer.

21. Use of angiogenesis inhibiting molecule as claimed in any one of the claims 10-13 for the preparation of a therapeutical or diagnostic composition for the treatment of solid tumors.

22. Therapeutical composition for the treatment of cancer, in particular for the treatment of solid tumors, comprising a therapeutically effective amount of one or more of the angiogenesis inhibiting molecules as claimed in any one of the claims 10-13 and an excipient, carrier or diluent.

23. Diagnostic composition for the diagnosis of cancer, in particular for diagnosing solid tumors, comprising a diagnostically effective amount of one or more of the angiogenesis inhibiting molecules as claimed in any one of the claims 10-13 and an excipient, carrier or diluent.

24. Monoclonal antibody H33 produced by hybridoma 13H33 as deposited on 22 October 2003 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the deposit accession number **DSM ACC2622**.

25. Fragments of monoclonal antibody H33 as claimed in claim 24.

26. Fragments as claimed in claim 25, wherein the fragments are selected from Fab fragments, Fv fragments, single domain antigen binding fragments, scFv and aggregates thereof, V_{HH}s.

27. Derivatives of antibody H33 as claimed in claim

24.

28. Derivatives as claimed in claim 27, wherein the derivatives are recombinant antibodies having the same specificity as antibody H33, humanized derivatives of antibody H33 and chimeric antibodies based on antibody H33.

29. Monoclonal antibody H33 as claimed in claim 24, fragments or derivatives thereof for use as a medicament.

30. Monoclonal antibody H33 as claimed in claim 24, fragments or derivatives thereof for use in the treatment or diagnosis of cancer.

31. Monoclonal antibody H33 as claimed in claim 24, fragments or derivatives thereof for use in the treatment or diagnosis of solid tumors.

32. Use of antibody H33 as claimed in claim 24, fragments or derivatives thereof for the preparation of a pharmaceutical composition for the treatment or diagnosis of cancer.

33. Use of antibody H33 as claimed in claim 24 for the preparation of a pharmaceutical composition for the treatment or diagnosis of solid tumors.

34. Therapeutical composition for the treatment of cancer, in particular for the treatment of solid tumors, comprising a therapeutically effective amount of antibody H33 as claimed in claim 24, fragments or derivatives thereof and an excipient, carrier or diluent.

35. Diagnostic composition for the diagnosis of cancer, in particular for detecting solid tumors in the human or animal body, comprising a diagnostically effective amount of antibody H33 as claimed in claim 24, fragments or derivatives thereof and an excipient, carrier or diluent.

36. Hybridoma 13H33 as deposited on 22 October 2003 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the deposit accession number DSM ACC2622.

ABSTRACT

The present invention relates to a method for providing molecules that are capable of inhibiting angiogenesis, comprising the steps of providing a range of molecules; testing whether these molecules can prevent interaction between JAM-B and JAM-C; testing the positive molecules for their ability to block angiogenesis *in vivo*; and selecting molecules that are positive in the angiogenesis test as angiogenesis inhibiting molecules. The method may further comprise the step of isolating or producing the angiogenesis inhibiting molecules. The invention further relates to the angiogenesis inhibiting molecules thus provided and produced, to their use in the treatment of cancer, to therapeutical and diagnostic compositions comprising them. In a particular embodiment the invention relates to monoclonal antibodies, in particular MAb H33, to soluble JAM-C and JAM-B and to small molecules.

Figure 1

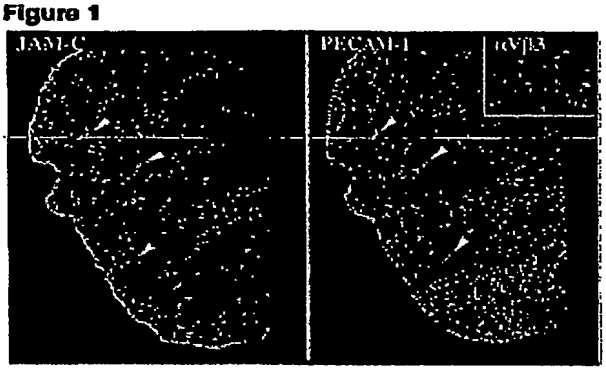


Figure 2

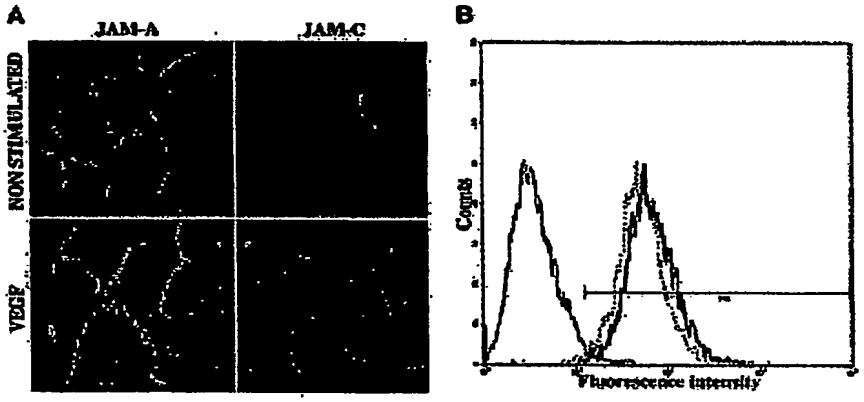


Figure 3

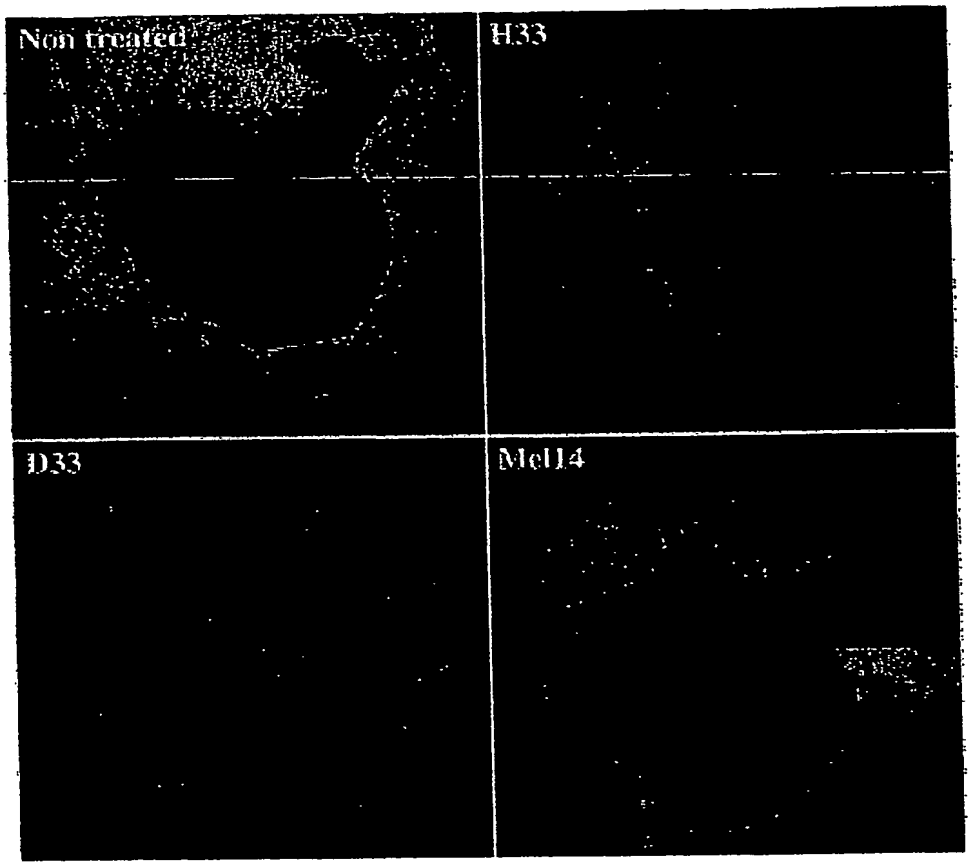


Figure 4

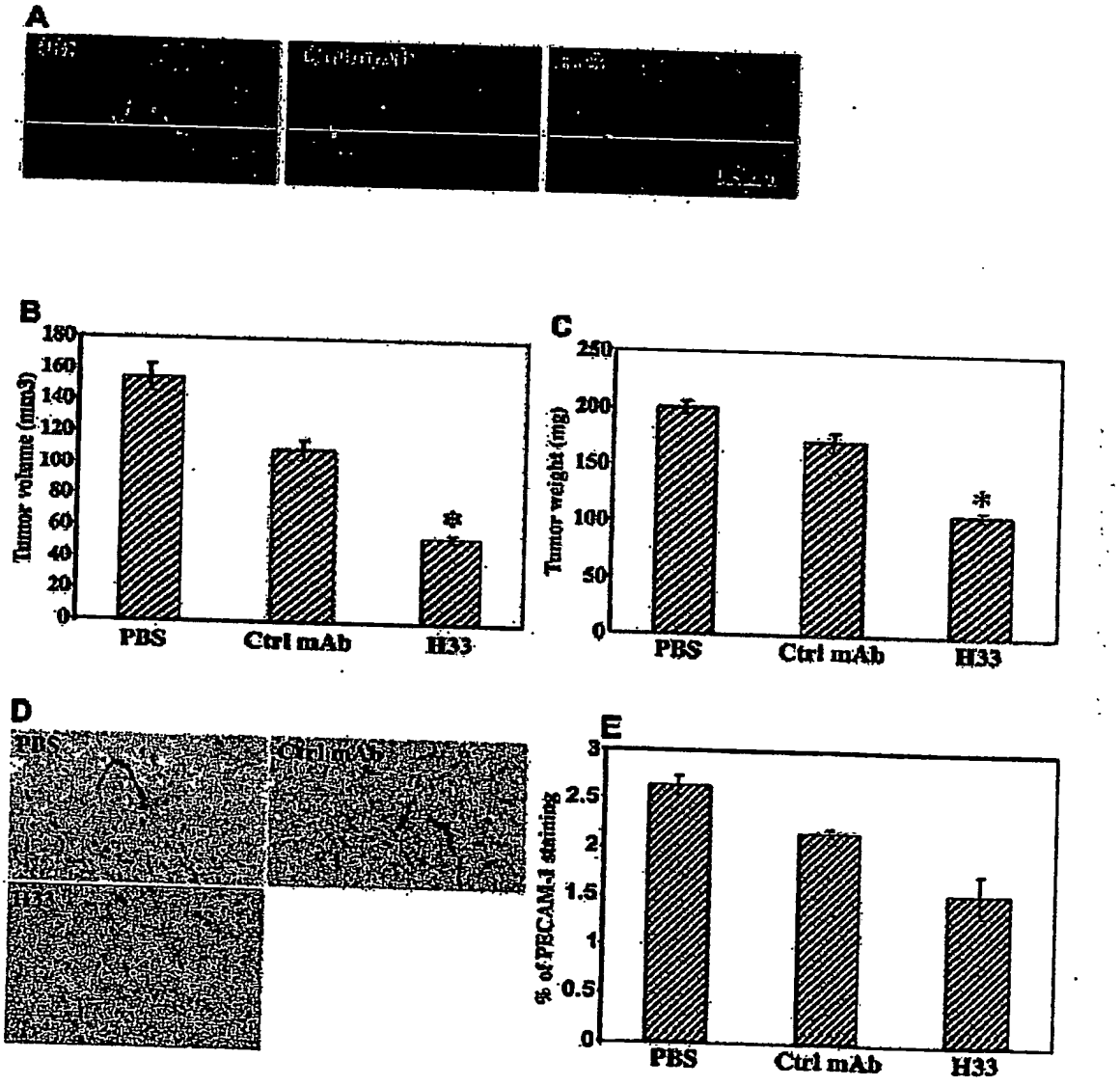
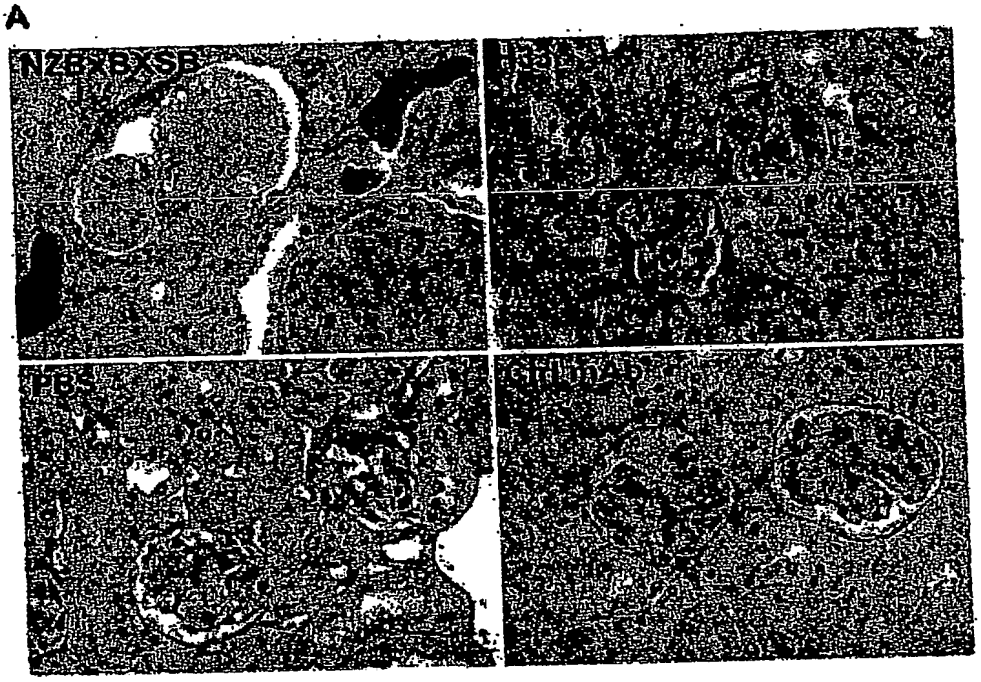
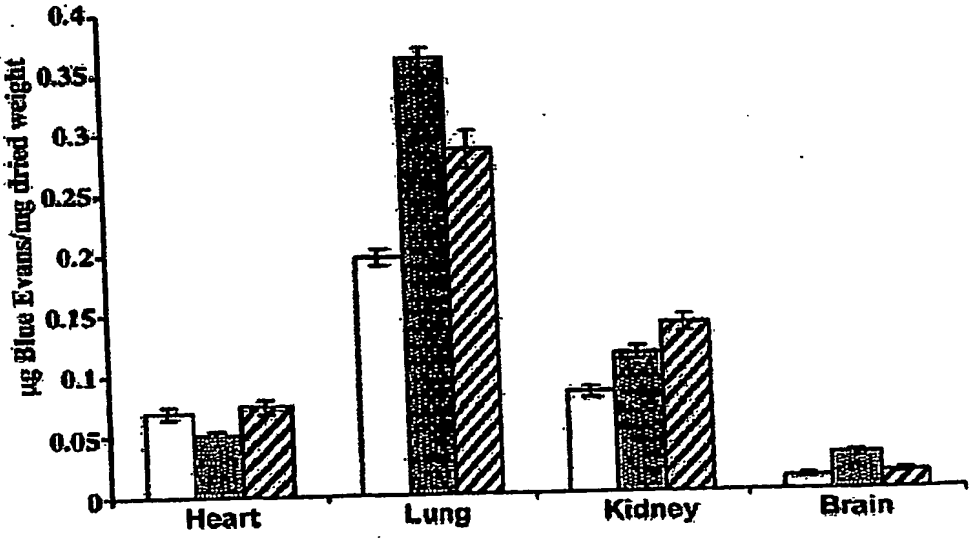

A
B
Tumor volume (mm3)
180
160
140
120
100
80
60
40
20
0
PBS
Ctrl mAb
H33
C
Tumor weight (mg)
250
200
150
100
50
0
PBS
Ctrl mAb
H33
D
PBS
Ctrl mAb
H33
E
% of PECAM-1 staining
3
2.5
2
1.5
1
0.5
0
PBS
Ctrl mAb
H33

Figure 5

A


NZBXBXSB
PBS
PBS

B


μg Blue Evans/mg dried weight
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Heart
Lung
Kidney
Brain

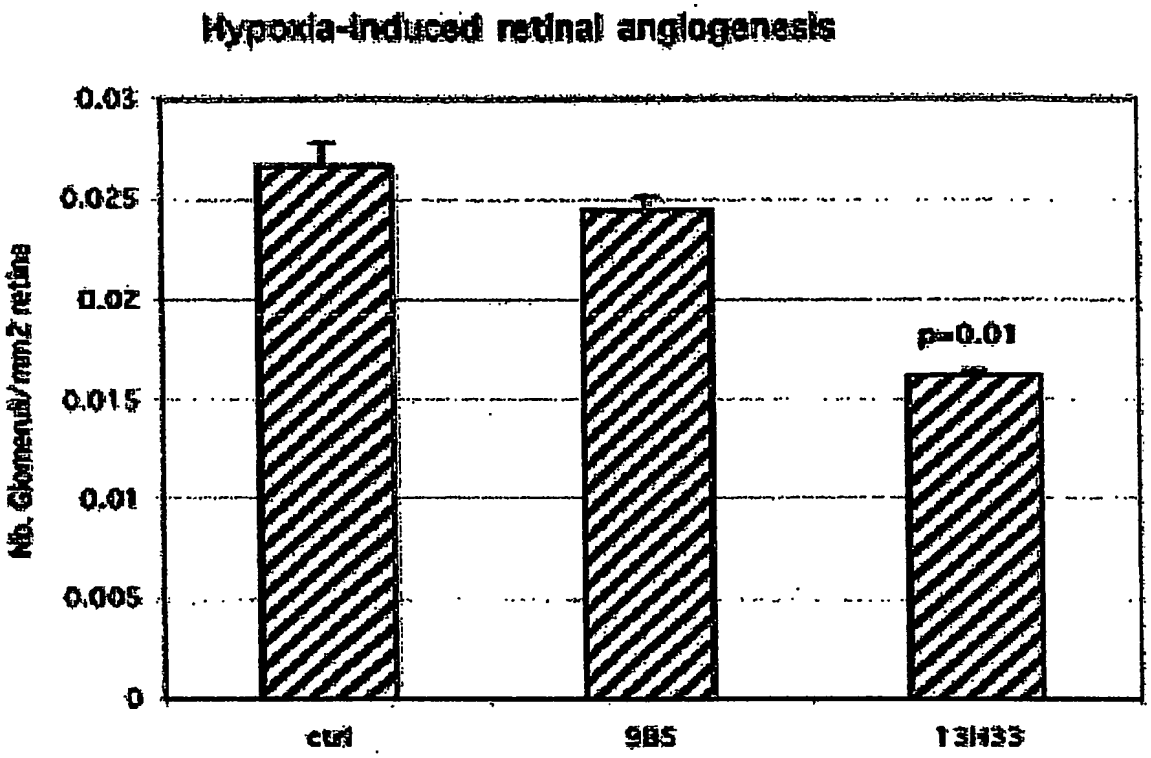

Hypoxia-induced retinal angiogenesis
Nb. Glomeruli/mm2 retina
0.03
0.025
0.02
0.015
0.01
0.005
0
p=0.01
ctrl
985
13H33

FIG. 6